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Preliminary crystallographic analysis of the *Megavirus* superoxide dismutase

Megavirus chilensis, a close relative of the *Mimivirus* giant virus, is able to replicate in *Acanthamoeba castellanii*. The first step of viral infection involves the internalization of the virions in host vacuoles. It has been experimentally demonstrated that *Mimivirus* particles contain many proteins capable of resisting oxidative stress, as encountered in the phagocytic process. These proteins are conserved in *Megavirus*, which has an additional gene (Mg277) encoding a putative superoxide dismutase. The Mg277 ORF product was overexpressed in *Escherichia coli*, purified and crystallized. A SAD data set was collected to 2.24 Å resolution at the selenium peak wavelength on the BM30 beamline at the ESRF from a single crystal of selenomethionine-substituted recombinant superoxide dismutase protein.

1. Introduction

Megavirus chilensis was isolated from an environmental sample from the Pacific Chilean coast (Arslan *et al.*, 2011); its natural host is unknown. *Megavirus* is an icosahedral virus of roughly 750 nm in diameter enclosing a 1.259 Mb double-stranded DNA genome encoding 1120 genes. It is clearly related to *Mimivirus*, with which it shares ~600 genes with an average of 50% sequence identity at the protein level (Arslan *et al.*, 2011; Legendre *et al.*, 2012). *Megavirus* was successfully isolated by co-cultivation with *Acanthamoeba castellanii*. As for *Mimivirus*, the first step of viral infection involves virion internalization in host vacuoles. The virion capsids present a specific structure coined the ‘stargate’ that is responsible for capsid opening in the phagosome and virion nucleoparticle transfer into the host cytoplasm by fusion of the virion and the phagosome membranes (Zauberman *et al.*, 2008). A transitory organelle, the virion factory, is seeded by the nucleoparticle in the host cytoplasm, where the entire replicative cycle takes place.

The proteomic study of purified *Mimivirus* particles highlighted the presence of many enzymes capable of resisting oxidative stress, as encountered in the phagosome (Badwey & Karnovsky, 1980; Brooks & Schneider, 1985). These enzymes are also encoded by the *Megavirus* genome, which has an additional gene encoding a possible superoxide dismutase (SOD). SODs catalyse the conversion of superoxide radicals to hydrogen peroxide and oxygen. There are three families of SODs, defined by their metal-ion-binding partners: Fe, Mn or Cu and Zn. The *Megavirus* Mg277 gene product seems to belong to the copper–zinc SOD protein family and shares ~50% sequence identity over 144 amino acids with eukaryotic enzymes.

The presence of such an enzyme in a virus is not unique. Poxviruses, for instance, also have a gene that is predicted to encode a copper–zinc SOD. However, the vertebrate poxvirus enzymes lack the key residues involved in metal binding necessary for catalytic activity and it has been demonstrated that the vaccinia virus protein (A45R) lacks any SOD enzymatic or inhibitory activity (Almazán *et al.*, 2001). The only known active viral SOD is that of the *Amsacta moorei* entomopox virus AMV255 (Becker *et al.*, 2004). The *Megavirus* Mg277 protein sequence presents all the residues necessary for metal coordination and catalytic activity. To verify that the *Megavirus* protein is a functional enzyme, we conducted a structural and functional study of the *Megavirus* copper–zinc superoxide dismutase.

2. Materials and methods

2.1. Expression of the Mg277 gene product

The gene encoding the *Megavirus* Mg277 protein (GenBank YP_004894328) was amplified from *Megavirus chilensis* genomic DNA and cloned by homologous recombination using an In-Fusion HD Cloning Kit (Clontech) according to the manufacturer's recommendations.

For PCR amplification, we used a forward primer (Mg277_F) containing the *Bam*HI site [16 nt homologous to the plasmid at (or upstream of) the *Bam*HI cloning site] followed by specific nucleotides of the 5' Mg277 gene (bold) and a reverse primer (Mg277_R) specific to the 3' sequence including the STOP codon of the Mg277 gene (bold) followed by the *Not*I site (17 nt homologous to the plasmid at the *Not*I cloning site): Mg277_F, CAGGGGCCCGGATCCTCAA-GATATGATTTTTTAAATGTAGTAACT, and Mg277_R, AAGC-ATTATGCGGCCGCTTAATTTAAATAAGCTATAATACCACAA-GC.

The PCR product was inserted by recombination in an in-house-modified pETDuet expression vector (Novagen), allowing the expression of protein fused with a His₆ tag to facilitate purification. A human rhinovirus 3C protease cleavage site allowed tag removal and facilitated the recovery of highly purified protein free of the classical *Escherichia coli* contaminants.

Expression was performed in *E. coli* Rosetta (DE3) transfected cells grown at 310 K in 2YT (Difco). Induction was performed at 290 K using 0.2 mM IPTG when the A_{600} reached ~1. The pellet from a 0.5 l overnight culture was resuspended in 40 ml 50 mM Tris-HCl, 300 mM NaCl buffer pH 8.5 (buffer A) containing 0.1% Triton X-100, 5% glycerol, 0.01% (w/v) DNase, 0.01% (w/v) lysozyme, three anti-

protease tablets (Roche Diagnostics) and total protein was extracted by sonication.

Since each monomer of the 161-amino-acid recombinant SOD protein contained two methionines, we decided to use the SAD method to solve the structure. Selenomethionine-substituted protein was produced using an appropriate protocol to inhibit methionine synthesis in the presence of selenomethionine and M9 minimal medium (Doublié, 1997). The protein was purified from 2 l cultures.

2.2. Purification

The cleared lysate was applied onto a 1 ml HisPur Ni-NTA column (Pierce) charged with Ni²⁺ and equilibrated with buffer A on an AKTÄexplorer 10S FPLC system (GE Healthcare) at a flow rate of 1 ml min⁻¹. The column was washed with ten column volumes of buffer A, ten column volumes of buffer A containing 25 mM imidazole and 20 column volumes of buffer A containing 50 mM imidazole at a flow rate of 1 ml min⁻¹. Elution was performed with a linear gradient from 50 to 500 mM imidazole over 20 column volumes at a flow rate of 3 ml min⁻¹.

The in-house-produced His-tagged human rhinovirus 3C protease was added to the pooled elution fractions followed by dialysis overnight at 277 K against 50 mM Tris-HCl buffer pH 8.5, 150 mM NaCl, 1 mM DTT, 0.1 mM EDTA.

A second run of affinity purification was performed to separate the cleaved Mg277 protein from uncleaved Mg277 and His-tagged protease, which were retained on the Ni-NTA column. The 161-amino-acid recombinant Mg277 protein corresponded to the native enzyme in which the initial methionine was replaced by a GPGS sequence resulting from the protease cleavage site.

After recovery from the flowthrough, the Mg277 protein was dialysed overnight in 20 mM Tris-HCl buffer pH 8 and concentrated using an Amicon Ultracel 10K centrifugal filter device (Millipore).

Gel filtration (performed on a Pharmacia Superdex S200 column) indicated that the Mg277 protein was dimeric in solution.

2.3. Crystallization

The selenomethionine-substituted recombinant Mg277 protein was concentrated to 6.9 mg ml⁻¹. Crystallization assays were performed at 293 K against 588 different conditions corresponding to commercially available solution sets (Crystal Screen from Hampton Research and Wizard from Emerald BioStructures) and in-house-designed conditions using the *SAmBA* software (Audic *et al.*, 1997). Screening for crystallization conditions was performed in 3 × 96-well crystallization plates (Greiner) loaded by an eight-needle dispensing robot (Tecan WS 100/8 workstation modified for our needs), mixing 0.5 µl protein solution with 0.5 µl reservoir solution in a sitting drop (Abergel *et al.*, 2003).

Crystals appeared in 0.1 M sodium acetate buffer, 20% (w/v) PEG 4000, 20% (v/v) MPD, 0.1 M ammonium sulfate adjusted to pH 5.5. We refined these conditions at 293 K by hanging-drop vapour diffusion using 24-well culture plates (Greiner). Each hanging drop was prepared by mixing 2 µl 6.9 mg ml⁻¹ Mg277 solution with 0.5 µl reservoir solution. The hanging drop on the cover glass was vapour-equilibrated against 1 ml reservoir solution in each well of the tissue-culture plate. We used increasing PEG 4000 concentrations (17–23%), 20% MPD with and without 0.1 M ammonium sulfate. The best crystals appeared after a few days without ammonium sulfate and reached typical dimensions of 0.4 × 0.15 × 0.1 mm (Fig. 1a). Data were collected from a crystal grown by mixing 2 µl selenomethionine-substituted Mg277 protein solution with 0.5 µl reservoir solution

Figure 1
Photograph (a) and diffraction image (b) of an Mg277 crystal.

Table 1

X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

Beamline	BM30A, ESRF
Wavelength (Å)	0.9793
Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters at 100 K (Å, °)	$a = b = 70.63$, $c = 117.43$, $\alpha = \beta = \gamma = 90$
Resolution range (Å)	45.96–2.24 (2.42–2.24)
Observations	221392 (45793)
Unique reflections	27389 (5642)
Multiplicity	14.9 (15.3)
Completeness (%)	100 (100)
Anomalous completeness (%)	100 (100)
$\langle I/\sigma(I) \rangle^\dagger$	12.6 (3.44)
$R_{\text{merge}}^\ddagger$ (%)	11 (63.2)

$^\dagger \langle I/\sigma(I) \rangle$ is the mean signal-to-noise ratio, where I is the integrated intensity of a measured reflection and σ is the estimated error in the measurement. $^\ddagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the integrated intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the mean recorded intensity of reflection hkl over multiple recordings.

consisting of 0.1 M sodium acetate buffer, 20%(w/v) PEG 4000, 20%(v/v) MPD adjusted to pH 5.5.

2.4. Data collection and processing

The crystal was collected in a Hampton Research 0.2×0.2 mm loop, flash-cooled to 105 K in a cold nitrogen-gas stream and subjected to X-ray diffraction; it diffracted to a resolution of 2.24 Å (Fig. 1*b*). Since we wanted to use the SAD method (Hendrickson & Teeter, 1981), a fluorescence scan was performed to determine the peak energy and a data set was collected on the BM30 beamline at the European Synchrotron Radiation Facility (ESRF), Grenoble, France at 12.662 keV. Data collection was performed with an oscillation angle of 0.5° and a crystal-to-detector distance of 291.72 mm, and 380 images were collected on an ADSC Q315r CCD detector. The crystal belonged to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 70.63$, $c = 117.43$ Å. The packing density for two monomers of Mg277 (17 690.8 Da) in the asymmetric unit of the crystal was $2.07 \text{ Å}^3 \text{ Da}^{-1}$, indicating an approximate solvent content of 40.55% (Matthews, 1968). Data statistics are presented in Table 1.

XDS (Kabsch, 2010) and SCALA (Evans, 1997, 2006) from the CCP4 package (Winn *et al.*, 2011) were used for processing, scaling and reduction of the data sets.

3. Results and discussion

Most viruses infect their hosts by simply injecting their genetic material through the cellular membrane and then use the host transcription and replication machinery for their replication. The *Megaviridae* infection cycle only takes place in the host cytoplasm and thus relies on the viruses' own complex transcription/replication machinery, which needs to be transferred simultaneously together with the viral genome. The peculiar mode of infection of *Megaviridae* makes this possible. It is a two-step process: firstly, virions enter the cell by fooling the amoeba into feeding on the giant virions as if they were bacteria. The virions are then confined to the phagocytic vacuole until an unknown signal triggers the opening of a specific structure at one vertex of the capsid: the stargate (Zauberman *et al.*, 2008). The more external of the two lipid membranes lining the virion

inner compartment is then able to fuse with the host phagosome membrane and create a continuum between the virion and the host cytoplasm. The internal membrane encloses the nucleoparticle, consisting of DNA and proteins, which is then transferred into the host cytoplasm, where it can begin transcription and initiate the early virion factory (Mutsafi *et al.*, 2010; Claverie & Abergel, 2009). Even if the precise composition of the *Acanthamoeba* digestive vacuole is unknown, it must contain everything needed for food digestion (proteases, oxidative agents). Interestingly, the *Mimivirus* proteome revealed the presence of many enzymes capable of resisting environmental stress such as oxidoreductases, thioredoxins, glutaredoxins and DNA-repair enzymes (Renesto *et al.*, 2006; Claverie *et al.*, 2009). The *Megavirus* genome surprisingly encodes an additional copper-zinc SOD enzyme that is absent from the *Mimivirus* genome. We thus expressed the Mg277 protein and produced crystals of the viral SOD suitable for X-ray diffraction studies. The presence of two methionines in each of the 161-amino-acid monomers should be sufficient to determine the structure of the selenomethionine-substituted recombinant SOD protein dimer using the four Se atoms for phasing. The Mg277 structure will be compared with its cellular and viral homologues to obtain some clues on the origin of the *Megavirus* protein, and enzymatic studies will be performed to address the recombinant enzyme activity *in vitro*. The presence of an active superoxide dismutase in purified viral particle extracts will also be assayed.

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